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It is hereby confirmed that the attached documents are corresponding with the original pages of the international application, as identified on the following pages, filed under Article 10 of the Patent Cooperation Treaty (PCT) at the receiving office named below.

Berne, le 19 avril 2005

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293-2.B.WO

PCT REQUEST

Original (for **SUBMISSION**)

0	**For receiving Office use only**	
0-1	International Application No.	PCT/CH 2004/00206
0-2	International Filing Date	01. April 2004 (01. 04. 2004)
0-3	Name of receiving Office and "PCT International Application"	RO/CH-Demande international PCT

0-4	**Form - PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.150)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	Swiss Federal Intellectual Property Institute (RO/CH)
0-7	**Applicant's or agent's file reference**	293-2.B.WO
I	**Title of Invention**	MANUFACTURING AND USE OF CARRIERS SUITED FOR ELECTROPHYSIOLOGICAL MEASUREMENTS
II	**Applicant**	
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IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent	
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V	**DESIGNATIONS**		
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**		
VI-1	**Priority Claim**	NONE	
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	3	-
IX-2	Description	6	-
IX-3	Claims	1	-
IX-4	Abstract	1	✓
IX-5	Drawings	5	-
IX-7	TOTAL	16	

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	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-17	PCT-SAFE physical media	-	✓
IX-19	**Figure of the drawings which should accompany the abstract**	1	
IX-20	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**		
X-1-1	Name:	ROLAND, André	
X-1-2	Name of signatory		
X-1-3	Capacity		

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10-1	**Date of actual receipt of the purported international application**	01. April 2004 (01. 04. 2004)
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	~~Not received~~	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	X

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11-1	**Date of receipt of the record copy by the International Bureau**	

MANUFACTURING AND USE OF CARRIERS SUITED FOR ELECTROPHYSIOLOGICAL MEASUREMENTS

Field of the Invention

This invention relates to methods and devices for analysis and detection systems based on artificial and biological (e.g. cell membranes) lipid membranes.

Background of the Invention

Many biological, physical or chemical analysis methods are based on lipid bilayers and biological membranes, respectively. Some of these techniques require the direct access to specific parts/patches of the membrane, which are usually in the range of 0.1 – 100 um (um .. 10^{-6} meter) diameter. Examples are electrophysiological techniques such as Patch Clamp and Black Lipid Membrane (BLM) analysis. Classically, such parts/patches of the membrane have been exclusively accessed e.g. by sealing a micro pipette against the cell membrane. Access to the membrane patch beneath the pipette is then directly provided through this pipette. The remaining membrane area outside the pipette is usually accessed through the solution in which the cell is immersed (B. Sakmann and E. Neher, Ed., *Single-Channel Recording*, Plenum Pub. Corp.; ed. 1, 1983). In the case of artificial lipid membranes (e.g. BLM), thin and perforated insulating sheets separating two fluid compartments have been used to carry the membranes in such a way that they cover the hole and consequently can be independently accessed on both sides (Mueller et al., J. phys. Chem., 67, 534 (1963)).

Lately, micromachined carriers made of sheets of insulating materials such as silicon/siliconnitride (PCT patent application WO1998IB0001150), glass and plastics have replaced the classical tools for directed membrane access such as micropipettes (as in patch clamp) and Teflon™ septa with conventional holes (as for BLM). Advantages include a much simplified handling during analysis, higher stability, better electrical parameters as well as the possibility to mass manufacture the new carriers.

However, due to the specific needs of carriers for electrophysiology such as the ability to easily manufacture holes as small as 0.1 – 20 um in ca. 2 – 200 um thick insulators, current standard techniques used for micromachining may not provide a suitable approach for the production of inexpensive but high quality membrane carriers. The use of controlled dielectric break down phenomena provides a new way of creating micro holes in insulating materials that serve as membrane/cell carrier.

Summary of the Invention

The present invention provides devices and methods for the creation of micro holes in insulating materials and their use for the independent access of at least two membrane areas and

consequently applicable as replacement of e.g. standard patch clamp pipettes and BLM septa. The invention uses the well known effect that at a critical electric field strength across insulating materials a dielectric break down (DEB) occurs which creates a track through this insulator. Using strong electric fields allows to also perforate thicker materials. Adjusting/limiting the current and time during the DEB controls the track thickness, which can be adjusted to be precise even for holes with less than 1 um diameter. Since both parameters, field strength and maximum current, can be independently controlled, tracks with high aspect ratios can be produced. Controlling the gas pressure and gas composition as well as the carrier properties (surface and bulk) during the DEB process provides the means for (quasi) simultaneous physico-chemical surface modification of the carriers due to the partial ionization of gas and surface components. This may be advantageous in cases where tight membrane adhesion to the carrier surface is required.

An important advantage of the described DEB methods and devices for controlled perforation is its applicability to most insulating materials. Because of the possible high aspect ratio of the produced track as well as the large choice of materials, membrane carriers with excellent electrical as well as membrane adhesion properties can be easily, quickly and inexpensively realised.

The foregoing and other advantages and features of the invention, and the manner in which the same are accomplished, will become more readily apparent upon consideration of the following detailed description of the invention taken in conjunction with the accompanying examples, which illustrate preferred and exemplary embodiments.

Brief Description of Figures

Figure 1 illustrates a typical embodiment of the device for DEB perforation of this invention;

Figure 2 illustrates a typical embodiment of the current-voltage control of the device for DEB perforation of this invention;

Figure 3 shows a microscopic image of a micro hole created in a thin polypropylene sheet and the corresponding current-time trace;

Figure 4 illustrates an embodiment of a device used for electrophysiological measurements with biological (lipid) membranes using the carrier device of this invention;

Figure 5 illustrates an embodiment of a device used for electrophysiological measurements with biological cells using the carrier device of this invention.

Detailed Description of the Invention

The device and methods of this invention can be used for the creation of hole and tunnel like structures in insulating materials, useful for electrophysiological and other measurements and set-ups where independent access to parts of biological membranes and cells is required.

The creation of high aspect ratio hole structures in insulating or semiconducting materials with current micromachining tools such as reactive ion etching or laser ablation is difficult, expensive and in most cases geometrically limited. However, for hole structures in insulating carriers used for the independent access of membrane parts, as e.g. patch clamp or BLM measurements, the precise location of the hole structure is less important compared to e.g. microelectronic circuits. Also, the hole diameter can vary largely (e.g. up to 100%) for the intended biological applications without significantly impacting the experimental quality and results. The possibility to create the hole like structure at a point that can be 'anywhere' at the carrier area reserved for membrane/cell access with only a roughly defined diameter provides the basis for the application of micro machining techniques that have lower precision than standard micromachining techniques.

A physical phenomenon that can be used to create small high aspect ratio holes, but lacks otherwise high precision required for e.g. microelectronics, is "dielectric break down" (DEB). This phenomenon occurs in insulators in electrical fields (e.g. sandwiched between two electrodes) when the applied voltage and electric field strength, respectively, increases to values where an "insulator-to-conductor" transition occurs. Due to Ohm's law $I = V \times R$ (I .. current, V .. voltage and R .. resistance), a sudden increase in current, and consequently power dissipation $P = R \times I^2$, between the electrodes and through the insulator is caused by the significant reduction in electrical resistance. Along the current path insulating material is transformed or removed ('burned') which can lead to the appearance of hole or tunnel like structures. This phenomenon is well known for decades and mostly a parasite effect in high voltage circuits or sensitive electronic components as e.g. FET transistors (gate electrodes). It has also been used in industrial environments to e.g. perforate thin plastic packaging sheets to permit gas exchange. Tests with DEB for the creation of small holes in plastic materials for BLM measurements have been tried in the past. Due to the employed devices which did not allow for a precise current and power control during and after DEB, micro holes with reproducible diameters below 20 um where not achieved. However, micro holes below ca. 20 um diameter are required for carriers for patch clamp like measurements (cell size usually < 25 um) and stable and commercially usable lipid membrane (e.g. BLM stability inverse proportional to membrane diameter) devices. Until now, DEB has never been used before in a defined and controlled manner for reproducible micro-structuring of insulating materials intended to carry small (i.e. less than ca. 20 um) biological membranes or objects thereof at the micro-hole site. In the following such objects will be called 'carrier'.

DEB structuring can be applied to essentially any insulating material, since all insulators show at some specific electric field strength a full or partial transition into a conducting state. Consequently, a wide selection of carrier materials exists allowing for an optimal selection of carrier parameters such as membrane and cell adhesion and electric/dielectric properties. Because the insulator-to-conductor transition field strength decreases with increasing insulator temperature, it

sometimes is recommended – in particular for materials where the break down point is difficult to achieve or side effects come into play – to insure an elevated temperature of the carrier during DEB.

To manufacture holes with a precise diameter by DEB the energy dissipation during and after DEB must be accurately controlled. Because the dissipated energy is the product of current x voltage x duration, all three factors are controlled. Figure 1 shows a possible realisation, in which the voltage is controlled by an adjustable and optionally current limiting high voltage power supply. Depending on the properties of the voltage source, the current may also be limited by an optional resistor R, which is in series with the carrier. The DEB duration is set by a timer which is triggered upon an adjustable current level indicating the onset of the DEB process. A possible realisation of a suitable high voltage source is illustrated in Figure 2. Figure 3 shows a micro hole of ca. 5 um created with DEB in a 20 um polypropylene sheet as well as the current-voltage trace recorded when the trans-carrier voltage was raised to the critical DEB value. Smaller holes (diameter < 1 um) were consistently produced by further limiting the current upon an increase in the series resistance R.

The distance between the electrodes and carrier to be structured can be varied. If the electrodes touch the carrier ('contact mode'), the necessary DEB voltage is minimal. However, contaminations and mechanical influences on the carrier deriving from the electrodes may occur. Using a gap between the carrier material and the electrodes increases the necessary DEB voltage, reduces however the risk of electrode interferences with the carrier surface. It provides however the means for a modification of the carrier surface through activated gas molecules. For this the gas composition between the electrodes and carrier is controlled in such a way that during DEB the ionized gas molecules interact with the carrier surface in a beneficial manner. An example is the usage of pure oxygen which leads to the generation of activated oxygen molecules/ions/radicals during DEB which can oxidize the carrier surface. Another way to concurrently modify the surface during DEB is the prior coverage of the surface with materials that upon the ionization and heating process during DEB undergo a chemical modification beneficial for the application of the carrier (e.g. better membrane adhesion). The surface properties of the DEB created hole and its surroundings can also be controlled by selection of a carrier material that during DEB is fully or in part transformed into a material of choice.

The electrodes can be surrounded by an insulating material such as PDMS that also tightly seals to the carrier surface. This avoids DEB bypassing the carrier and going through the adjacent medium (e.g. air) and consequently allows to structure also carriers with small surface areas. Another solution is the usage of carrier surrounding media that have a much higher break down voltage that the carrier material.

The combination of DEB micro-structured carriers with the means for electrophysiological measurements provides the basis for a new and inexpensive way to monitor electrical currents through biological membranes. Here the carrier separates two or more fluid compartments that are only

connected through the DEB produced hole. The biological membranes to be analysed are placed on one side of the carrier across the hole and sealing it tightly. Figure 4 illustrates the usage of a DEB micro structured carrier as support for an artificial lipid membrane in a BLM set-up. Figure 5 illustrates the usage of a DEB micro structured carrier as support for a patch clamp type set-up with biological cells. For such measurements it is required that membranes adhere tightly (forming so called 'giga seals') to the surface of the carrier thus avoiding leakage currents bypassing the biological membranes. Currents measured across the carrier are largely modulated by the behaviour of the hole spanning membrane.

Figure 1 is a schematic diagram (side view) illustrating an embodiment of a device for DEB based manufacturing of defined micro holes, consisting of the insulating carrier material to be structured (1) between electrodes (2); the electrodes can have various forms (2) and distances to the carrier material; the electrodes are connected to an adjustable high voltage source (3); an optional series resistance R (4) may be connected in series with the electrodes to limit the current during DEB. The voltage source may control the DEB process in such a way that the maximum current and the duration of current flow after DEB is precisely adjusted. The carrier material and electrodes may be surrounded by a controlled gas composition and pressure (5).

Figure 2 is a schematic diagram illustrating a possible embodiment of a current-voltage source for controlled creation of dielectric breakdown holes for carriers of biological membranes. The operator (1) sets via a computer (2) with attached digital-analog/analog-digital converter (3) the voltage (4) and maximum current (4) of controllable high voltage source (6) (e.g. EuroTest CPP300304245, Germany). Voltage is applied to the carrier (9) via electrodes (8) and an optional current limiting resistor (7). The resistor may be necessary when the internal current limitation of the voltage source does not respond quickly enough or large capacitances in parallel to the electrodes render the current limitation circuits of the voltage source inefficient for quick response. The current through the carrier (9) is monitored by the computer via a current monitoring signal (5) coming from the voltage source. Upon program driven voltage increase until dielectric breakdown occurs, a timer is triggered at DEB that limits the duration of the current flow. This consequently sets the electric energy, which is partially transformed into heat energy, going into the hole creation process and with it the hole diameter.

Figure 3 shows a microscopic image (upper picture) of a hole produced with DEB in a 20 um thick polypropylene (PP) sheet. The hole diameter is ca. 5 um. The lower part shows the current-voltage curve (uA – kV) recorded during DEB micro structuring. The parameters were:
R = 10 GOhm, V = 6.4 kV, Imax = 1.8 uA and the voltage was raised with dV/dt = 60V / 80msec. Voltage was lowered to 0 kV immediately upon DEB. Electrode distances to the PP sheet were ca. 10 – 200 um.

Figure 5 shows a possible realisation of a device using DEB micro structured carriers for electrical membrane measurements. The carrier (1) separates two fluid compartments having any shape and boundaries (8, 9) which are only connected through the DEB produced channel (2). One side of the channel is covered by a biological membrane (3). Upon tight binding of the biological membrane to the carrier surface voltages applied through the fluid immersed (redox) electrodes (4) lead to a current that is only dependent on the properties of the biological membrane itself. Current voltage measurements may be performed with a suitable device (5) allowing to set the voltage (6) and measure the current (7). For some electrophysiological measurements the device (5) may be substituted with a voltage measuring device.

Figure 6 shows a possible realisation of a device using DEB micro structured carriers for electrical membrane measurements on biological cells as e.g. patch clamp measurements. The carrier (1) separates two fluid compartments (6, 7) which are only connected through the DEB produced channel (2). One side of the channel is covered by a biological cell (3). Upon tight binding of the biological cell to the carrier surface voltages applied through the fluid immersed (redox) electrodes (4) lead to a current that is only dependent on the properties of the membrane of the cell itself. Upon removal of the membrane patch covering the hole, the almost entire remaining cell membrane contributes to the trans-carrier current (whole cell mode). Current voltage measurements may be performed with a suitable device (5), such as a patch clamp amplifier (e.g. Axon Instruments).

CLAIMS

1. A device consisting of an insulating carrier containing at least one hole, the hole being made by a DEB process and being of a controlled size so that it can be entirely covered by a biological cell or small biological membrane of less than 25 micrometer, separating at least two fluid compartments, which are accessed by electrodes, in such a way that the fluid compartments are only connected through the DEB produced hole in the carrier itself.

2. A process using DEB in a controlled manner, that is by control of voltage, current and time of the DEB, to produce small holes of a controlled size of less than 25 micrometer in insulating carriers, intended to be covered by biological cells or other biological membranes such as lipid bilayers.

ABSTRACT

The invention concerns a device consisting of an insulating carrier containing at least one hole, the hole being made by a DEB process and being of a controlled size so that it can be entirely covered by a biological cell or small biological membrane of less than 25 micrometer, separating at least two fluid compartments, which are accessed by electrodes, in such a way that the fluid compartments are only connected through the DEB produced hole in the carrier itself.

The invention also relates to a process for manufacturing said device.

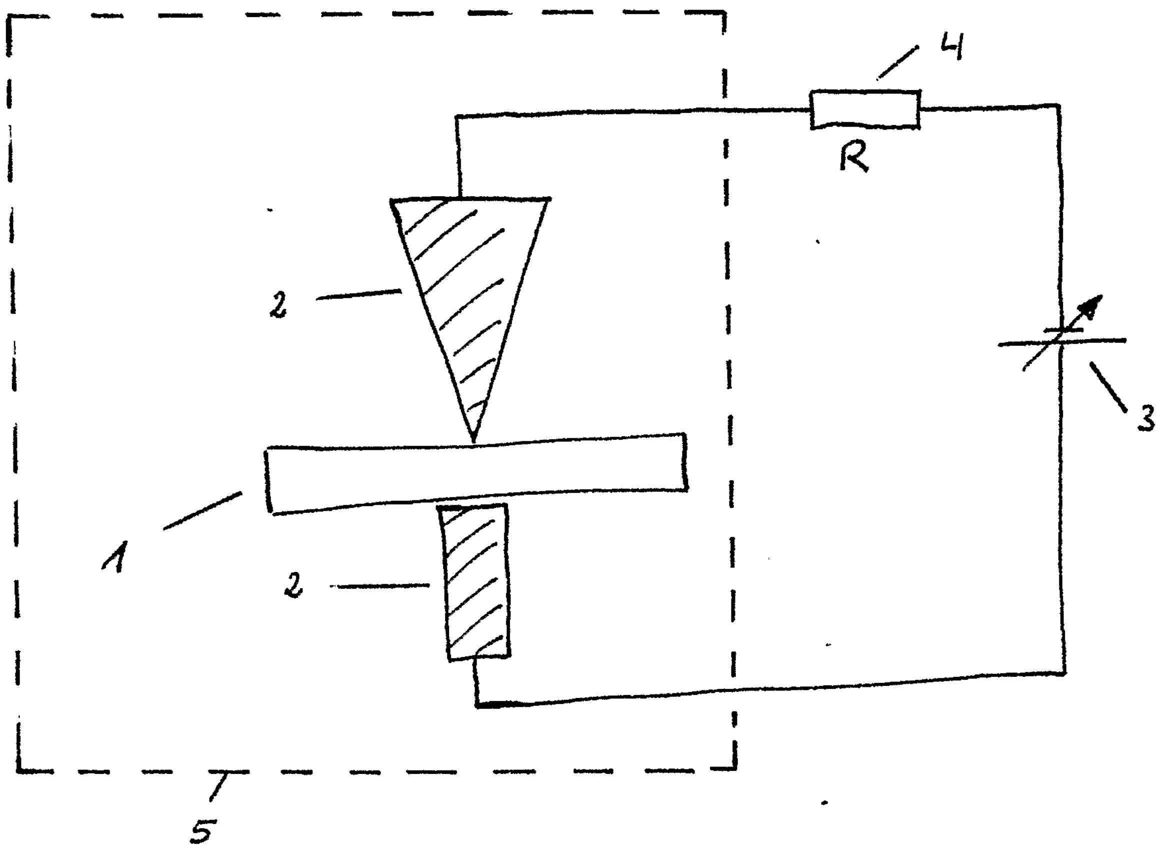


Figure 1

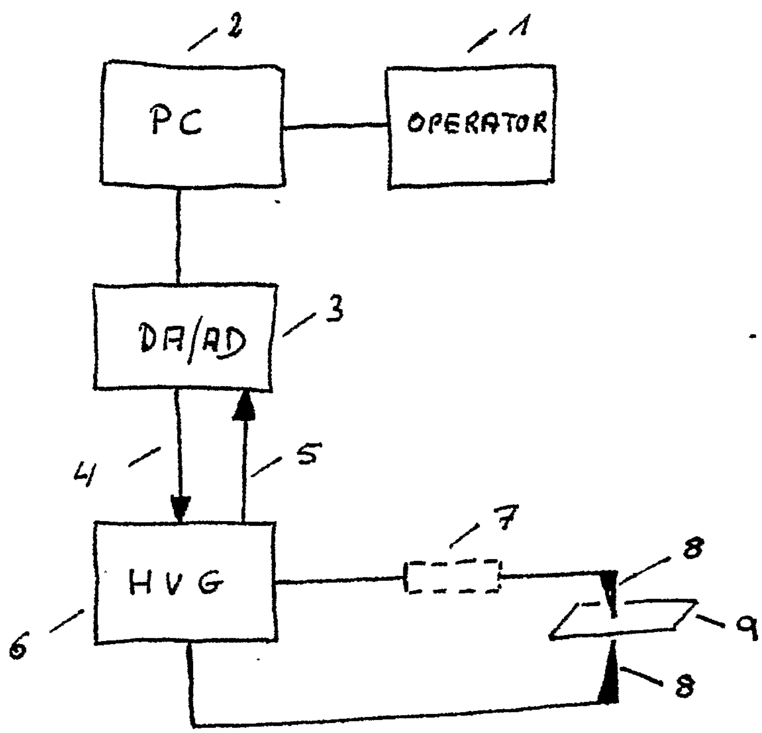
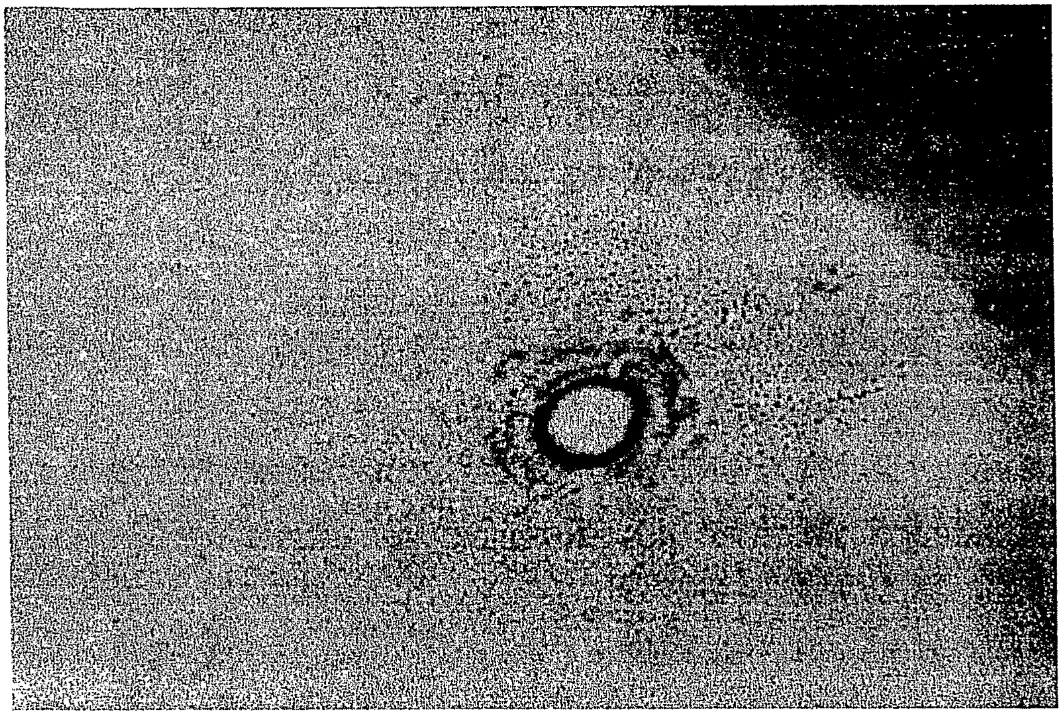


Figure 2



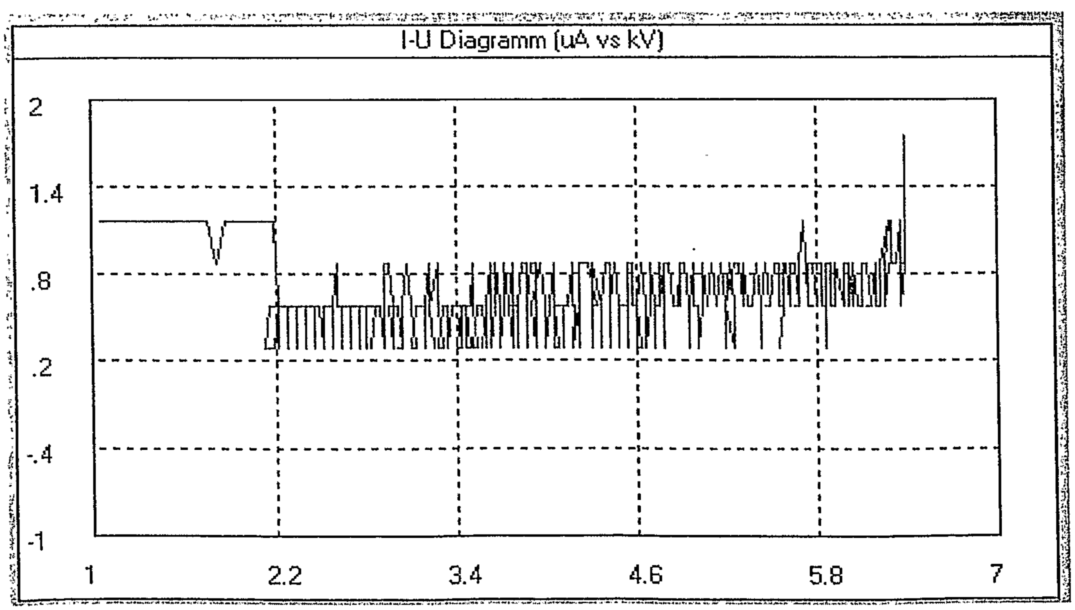


Figure 3

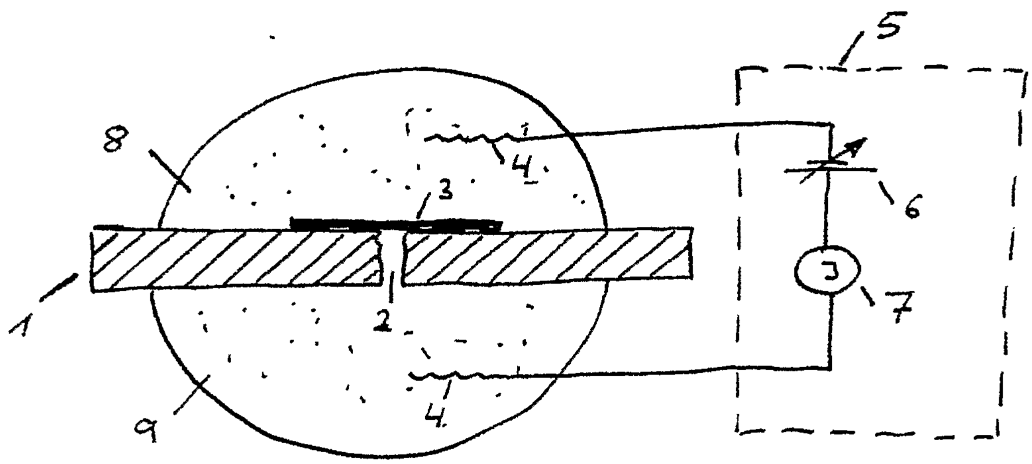


Figure 4

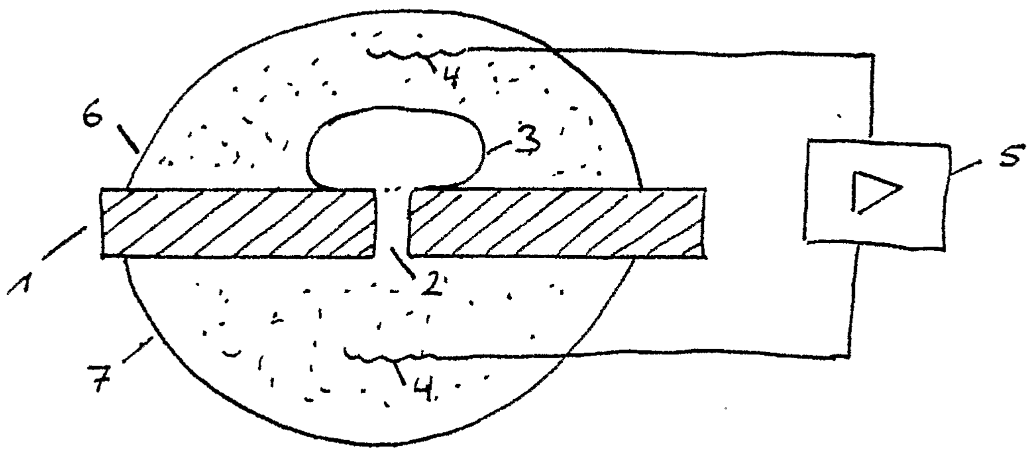


Figure 5